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Inocybe ahmadii sp. nov. and a new record of *I. leptocystis* from Pakistan

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Abstract—Two smooth-spored *Inocybe* species, *I. ahmadii* (a new taxon) and *I. leptocystis,* were collected from moist temperate forests of Pakistan and identified based on molecular phylogenetic analysis and assessment of macro- and micro-morphological characters. *Inocybe ahmadii* is described as new based on its smooth phaseoliform to sub-amygdaliform basidiospores, pinkish brown lamellae, white stipe surface that is brownish at the apex, broadly lageniform hyaline pleurocystidia, and similar but more frequent cheilocystidia. Our *I. leptocystis* specimen, molecularly supported as conspecific with specimens from Austria, China, Italy, and Finland, represents a first record of the species from Pakistan.

Key words—conifers, *Inocybaceae*, *Inocybe* sensu stricto, *Inocybe* subg. *Inocybe*, nrITS

Introduction

Inocybe (*Basidiomycota, Agaricales, Inocybaceae*) is a highly diverse genus of ectomycorrhizal fungi comprising 500–750 species, primarily in temperate areas. The number continues to increase due to new discoveries (e.g., Matheny et al. 2009, Alvarado et al. 2010, Bougher & Matheny 2011, Bougher et al. 2012, Kokkonen & Vauras, 2012, Matheny et al. 2012, Fan & Bau 2013, Braaten et al. 2014, Fan & Bau 2014, Latha et al. 2015, Esteve-Raventós et al. 2015, Jabeen et al. 2016). Twenty-eight species of *Inocybe* have been reported from Pakistan (Ahmad et al. 1997, Sultana et al. 2011, Saba et al. 2015, Jabeen et al. 2016).

The species within *Inocybe* can be recognized by their often small- to medium-sized basidiomata with spermatic, earthy, bitter almond, pelargonium, or fruit-like smell (although many species do not have any distinct smell), radially fibrous to cracked and often brownish pileus, and smooth, spinose, nodulose, or angular yellowish brown basidiospores (Solak et al. 2009). Matheny et al. (2009) subdivided *Inocybe* into seven major clades or lineages based on DNA sequence analyses and morphology.

Taxa within *Inocybe* subg. *Inocybe* [= *Inocybe* s.str.] typically have angular/nodulose or smooth spores, thick-walled pleurocystidia, a cortina, and a non- to only slightly pruinose stipe apex. Our study characterizes and identifies two species within *Inocybe* s.str. based on morphological and internal transcribed spacer (ITS) nrDNA analyses.

Materials & methods

Sampling site

Sampling was carried out during the 2014 and 2015 monsoon periods in Khanspur and Miandam of Khyber Pakhtunkhwa province, Pakistan.

Miandam is located in northeastern Swat district, a lush green valley surrounded by mountain peaks ranging from 4500 to over 6000 m a.s.l. and mostly covered with permanent snow. The region has a humid climate with average annual rainfall exceeding 1000 mm. Summers are mild: the hottest month is June, with mean 33°C maximum and 16°C minimum temperatures, and a rainfall of 120–132 mm in July–August. January, with mean −2°C minimum and 11°C maximum temperatures, is the coldest month (Sher et al. 2012).

Khanspur in Abbottabad district lies in the moist temperate western Himalayan mountain range (3000–4000 m a.s.l.; Khan 1999). The mean annual temperature is 10°C (Malik & Sukhera 2012). The area is characterized by cool summers and cold winters, with a 3°C mean winter temperature during December and January and 26°C mean summer temperature during June to September; mean annual rainfall is 1200 mm with 57% humidity (Ahmed et al. 2006).

The vegetation of both regions is dominated by coniferous trees (*Cedrus*, *Picea*, *Pinus*, *Taxus*) with deciduous trees (*Juglans*, *Populus*, *Quercus*, *Salix*) also present (Champion et al. 1965; Sheikh 1993)

Collection, isolation, and morphological study

Basidiomata were collected, tagged with their assigned sample code and collection date, and photographed. Fresh macroscopical characters were documented in detail. Colors were designated following the Munsell Soil Color Charts (1975). For subsequent anatomical and molecular analyses, specimens were dried under an electric fan heater to prevent any potential fungal attack or decomposition. Tissues were mounted in 5% KOH and spores, basidia, cystidia, pileipellis, and stipitipellis were examined under a MX4300H light microscope (Meiji Techno Co., Ltd., Japan). Line drawings of microscopic characters were made with a Leitz camera lucida (Wetzlar, Germany). For each species, 20 basidiospores / from 1 specimen / from 1 collection (indicated as: [20/1/1]) were measured. Dimensions are given as (a–)b–c(–d), with the range b–c containing at least 90% of the measured values and extreme values (a & d) given in parentheses. Abbreviations: avl = average spore length, avw = average width, Q (quotient) = spore length/width ratio, and avQ = average quotient of all basidiospores (Liang & Yang 2011). Specimens were deposited in the Herbarium, University of the Punjab, Lahore, Pakistan (LAH).

DNA extraction, amplification & sequencing

Genomic DNA was extracted from a small piece (30–50 mg) of dried pileus and lamellae using 2% CTAB extraction buffer following the Bruns (1995) protocol with some modifications (300 µl of chloroform was combined with iso-amylalcohol (24:1), centrifuge conditions altered to 13.2 rpm for 20 min at 4°C, and the DNA pellet was resuspended in 50 µl of deionized water). The nrDNA ITS region was amplified with ITS1F as forward primer and ITS4 as reverse primer (White et al. 1990) using the Extract-N-Amp PCR kit (Sigma Aldrich, St. Louis, MO, U.S.A.). The DNA was amplified in 20 µl reaction volumes following thermocycler conditions in Gardes & Bruns (1993). Agarose gel electrophoresis was performed to visualize amplified fragments of DNA in the Uvtec gel documentation system (Avebury House, Cambridge, UK) using default settings (Sambrook & Russell 2001). PCR products were directly sequenced bi-directionally using the same primer set at Macrogen Inc., Republic of South Korea.

Molecular phylogenetic analysis

The generated sequences were assembled using BioEdit software. Nucleotide sequences were compared with the Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI; U.S.A. database). Closely related sequences with identity greater than 90% and maximum query cover with 0.0 E value were retrieved from GenBank to reconstruct an ITS phylogeny. All sequences were aligned using MUSCLE (http://www.ebi.ac.uk/tools/msa/muscle). The alignment portion from CATTA to GACCT between the conserved motifs GAT to CAAA was included in analysis. MEGA6 (Tamura et al. 2013) was used for phylogenetic tree construction. A Maximum Likelihood (ML) tree of nrITS sequences was constructed using a General Time Reversible (GTR) model and nearest-neighbor-interchange (NNI) as a ML heuristic search method. 1000 bootstrap replicates were performed.

Inocybe ahmadii Farooqi, Niazi & Khalid, **sp. nov.** FIGS 1, 2

MYCOBANK MB 817163

Differs from *Inocybe glabripes* by its lageniform pleurocystidia and numerous lageniform cheilocystidia.

TYPE: Pakistan, Khyber Pakhtunkhwa province, Abbottabad district, Khanspur, 3000–4000 m a.s.l., solitary on ground under *Pinus wallichiana* A.B. Jacks., 14 August 2015, Ayesha Farooqi AF14 (**Holotype**: LAH14215; GenBank KX254462).

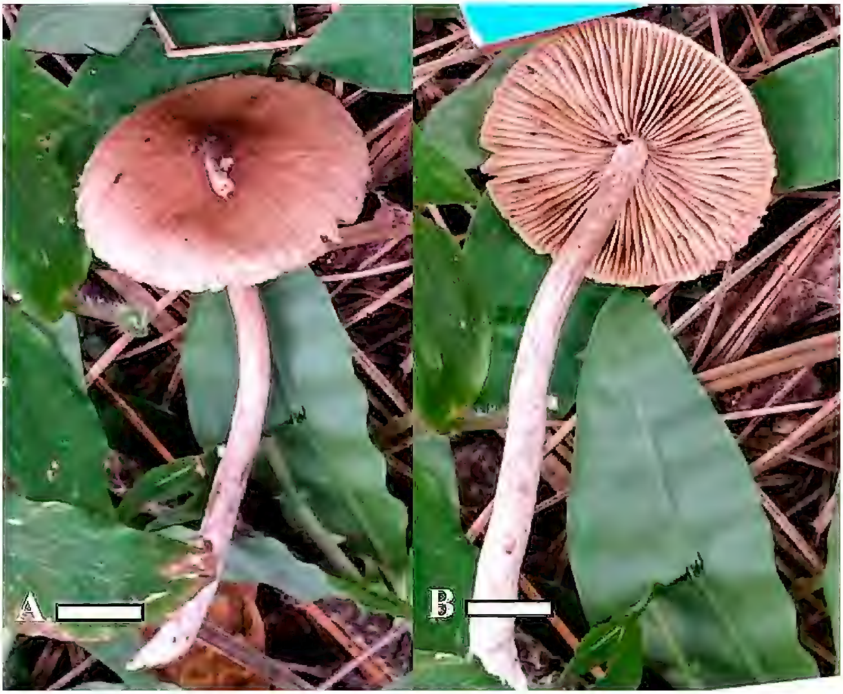

Fig. 1. *Inocybe ahmadii* basidiome (LAH14215, holotype). Scale bars = 1 cm.

Etymology—The specific epithet refers to Sultan Ahmad, the pioneer mycologist of Pakistan, for his contributions in the field of mycology.

Basidioma small, ≤46 mm high, pale brown when dry. Pileus small, 21 mm diam, plano-convex to applanate, umbonate, umbo rather low and obtuse; dark pinkish brown (5YR1/4) at the centre, lighter towards margins, light pinkish brown (5YR4/6); surface dry, with appressed scales, weakly fibrillose towards the margin, slightly rimulose, edges appendiculate with whitish fibrils; context very thin, whitish. Lamellae adnexed, moderately crowded, alternating with lamellulae, 0.1 mm wide, buff (7.5YR9/4), turning brown when dry, edges entire to slightly fimbriate. Stipe 42 × 3 mm, central, even, surface pruinose at the apex, fibrillose towards the base, brownish at the apex, white to greyish towards the base, cortina present and fugacious. Context loosely stuffed, interior tubular. Odour not recorded.

Basidiospores [20/1/1] (7.8–)7.9–9.0(–9.2) × (4.3–)4.7–4.9(–5.0) µm, avl × avw = 8.4 × 4.7 µm, Q = (1.5–)1.6–1.8(–2.0), avQ = 1.7, ellipsoid; phaseoliform

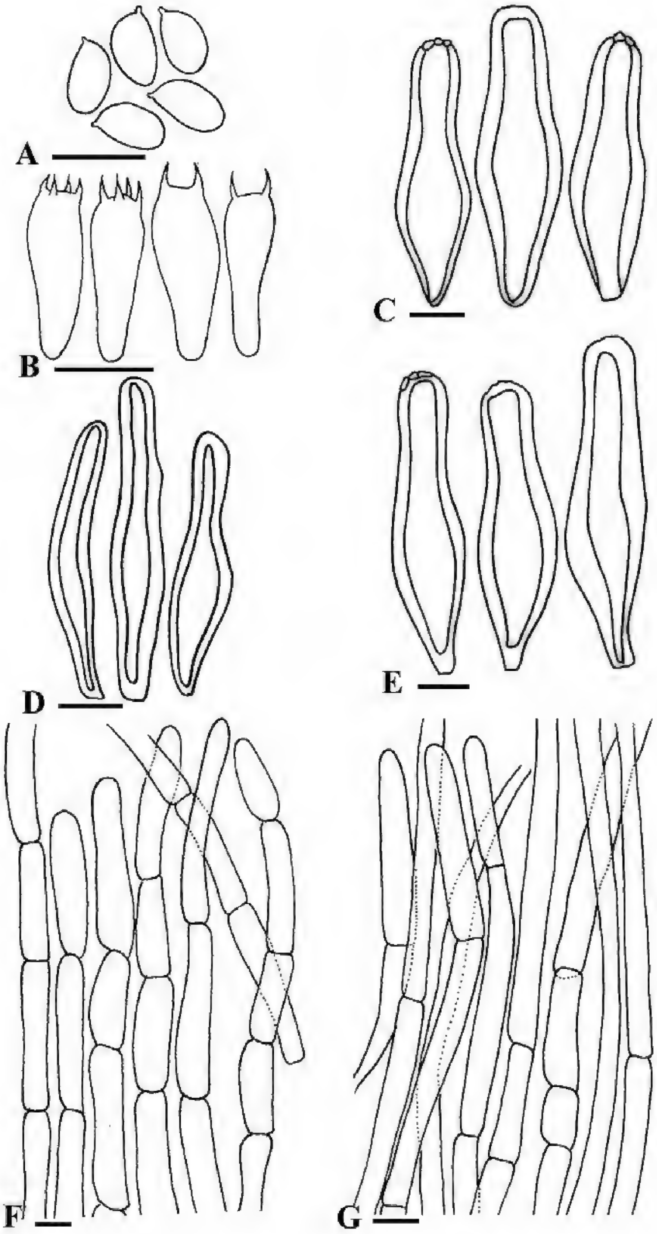

Fig. 2. *Inocybe ahmadii* (LAH14215, holotype). A. basidiospores; B. basidia; C. cheilocystidia; D. pleurocystidia; E. caulocystidia; F. hyphae from pileipellis; G. stipitipellis Scale bars: A–E = 20 µm; F,G = 40 µm. Drawings by Ayesha Farooqi.

to subamygdaliform, apiculate, mostly with obtuse apices, pale yellow brown, uniguttulate. BASIDIA 19.5–25 × 6.5–8.5 µm, avl × avw = 22.5 × 7.5 µm, cylindrical to clavate, 2–4-spored, hyaline. PLEUROCYSTIDIA 51.5–65 × 12–17.5 µm, avl × avw = 59 × 14.5 µm, narrowly to rather broadly lageniform with obtuse apices and a small pedicel, wall ≤3 µm thick. CHEILOCYSTIDIA 49–55 × 13–16.5 µm, avl × avw = 52 × 15 µm, similar to pleurocystidia but generally shorter and wider, moderately encrusted at the apex, rarely tapering at the base, hyaline, paracystidia rare. CAULOCYSTIDIA slender, cylindrical, rarely subfusiform, moderately thick-walled, present only at stipe apex. PILEIPELLIS 9–24 µm thick (av = 12.5 µm), a trichoderm, hyphae ochraceous, clamp connections present. STIPITIPELLIS 6–14 µm thick (av = 9.0 µm), composed of cylindrical hyphae, regularly arranged, thin-walled, clamp connections present.

Inocybe leptocystis G.F. Atk., Am. J. Bot. 5: 212 (1918) FIGS 3,4

PILEUS 16 mm diam, yellowish brown (5YR5/8) to brown (5YR9/12) towards the margin, smooth to finely fibrillose, umbonate, margins incurved. LAMELLAE moderately wide, adnate, 2–4 mm wide, light brown (2.5YR2/6), edges fimbriate. STIPE 45 × 7 mm, cylindrical, brown (2.5Y3/6) to whitish (2.5YR9/12), slightly enlarged at the base; surface smooth, nowhere pruinose; central, narrower towards the apex. CONTEXT white to yellowish towards the stipe base. ODOUR weakly spermatic.

BASIDIOSPORES [20/1/1] (9.0–)10.1–12.5(–12.7) × (5.2–)5.4–6.4(–6.6) µm, avl × avw =11.6 × 6.0 µm, Q = (1.5–)1.7–2.2(–2.3), avQ = 1.9, smooth, subamygdaliform, yellowish brown, apiculate, sometimes slightly depressed above the apiculus, mostly uniguttulate. BASIDIA 28.3–35.4 × 7.1–9.2 µm, avl × avw = 33.2 × 8.6 µm, cylindrical, hyaline to yellow, 4-spored. PLEUROCYSTIDIA 35–60 × 16–23 µm, avl × avw = 53 × 18 µm, with median constriction and clavate to utriform, apices obtuse or subcapitate, thin-walled, hyaline to light yellow. CHEILOCYSTIDIA 52–68 × 12–24 µm, avl × avw = 62 × 17.5 µm, clavate, thin-walled, hyaline to light yellow, in various forms, rather dispersed. CAULOCYSTIDIA not observed. PILEIPELLIS 6–10.5 µm thick (av = 9.8 µm), a cutis, hyaline, composed of irregular hyphae, walls yellowish brown, clamp connections present. STIPITIPELLIS 7–9 µm thick (av = 8.2 µm), a cutis of cylindrical hyphae, yellowish brown pigment incrusted, septate.

MATERIAL EXAMINED: **PAKISTAN, KHYBER PAKHTUNKHWA PROVINCE, Swat District,** Miandam at 2000 m a.s.l, under conifers, solitary, 23 Aug 2014, Sana Jabeen FA-5 (LAH35112; GenBank KX254461).

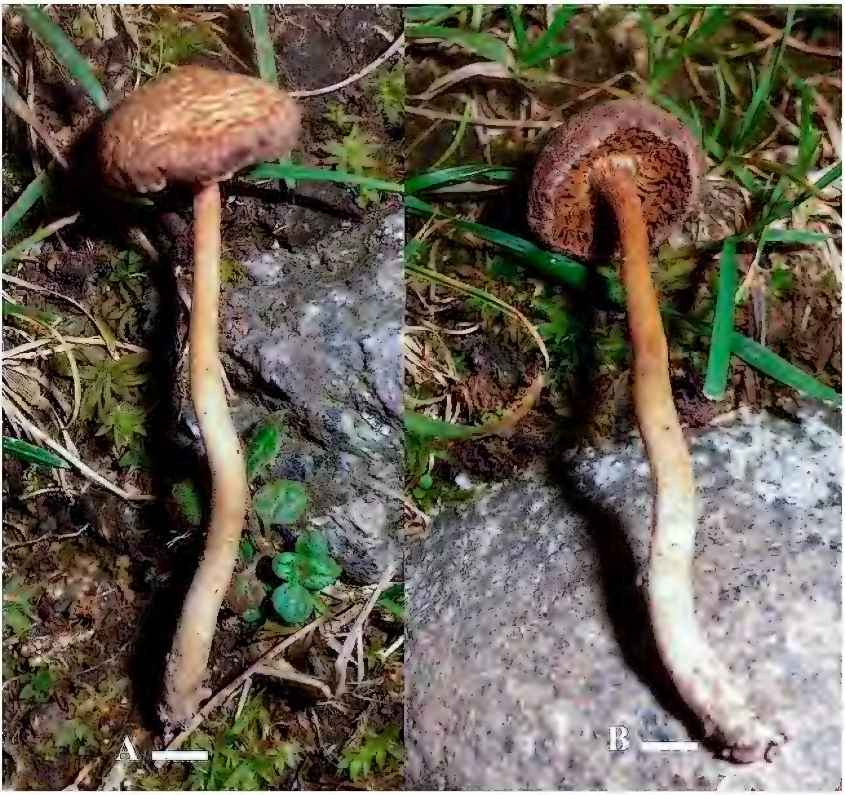

Fig. 3. *Inocybe leptocystis* basidiome (LAH35112). Scale bars = 0.5 cm.

Molecular phylogenetic results

Sequences of the *Inocybe ahmadii* PCR products using ITS1F and ITS4 were trimmed at the conserved motifs to produce consensus sequences of 643 base pairs. BLAST searches of *Inocybe ahmadii* ITS1F and ITS4 sequences revealed 93–91% identity with *Inocybe* sp. (JF908230) from Italy listed as "*Inocybe rufulovelata*", an unpublished name, in Osmundson et al. (2013) and *I. glabripes* Ricken (AJ889952) from Denmark with 94–100% query cover and 0.0 E value.

Sequencing of the *Inocybe leptocystis* PCR product using the same set of primers yielded 727–809 base pairs, which were trimmed at the conserved motifs to produce consensus sequences of 637 base pairs. The BLAST revealed a 99% similarity with *I. leptocystis* from China (KU359777) and Italy (JF908158) and a 95–94% similarity with a different species identified as *I. leptocystis* from China (KJ810596) and Sweden (AM882801). It therefore appears that the label

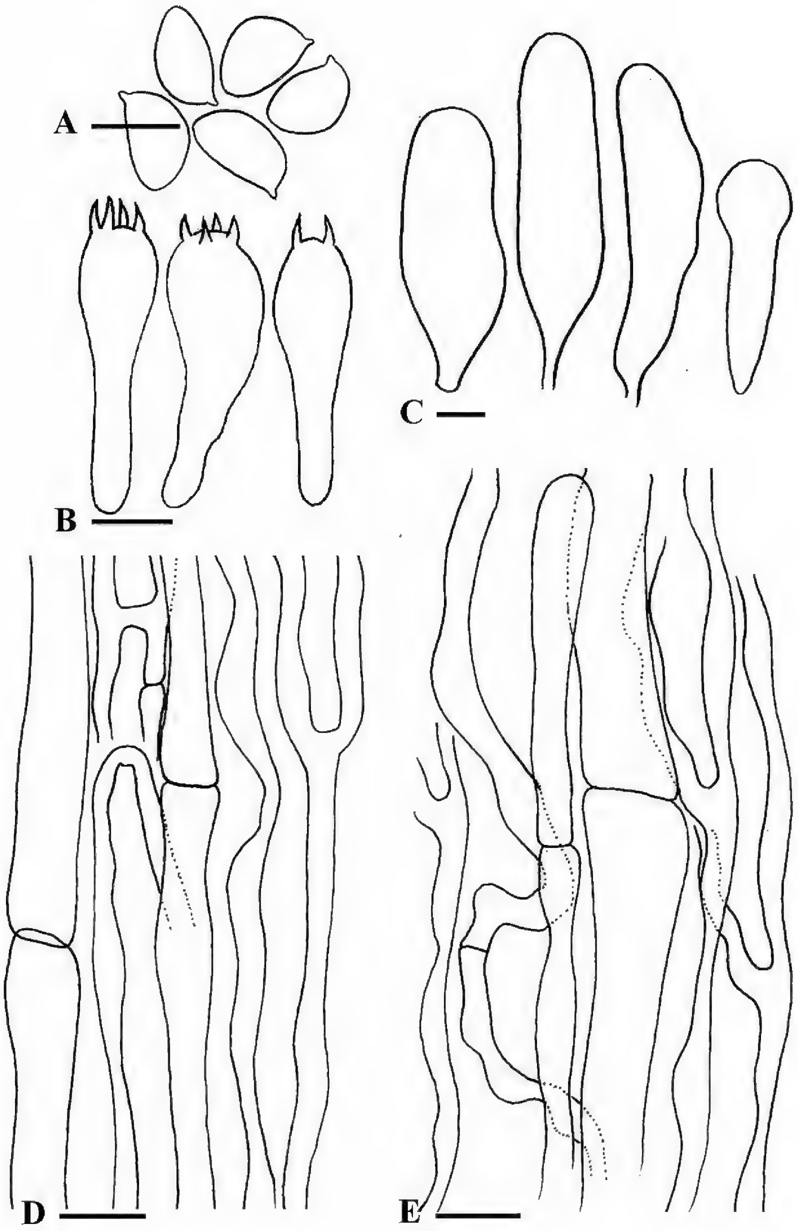

Fig. 4. *Inocybe leptocystis* (LAH35112). A. basidiospores; B. basidia; C. pleurocystidia; D. stipitipellis; E. pileipellis. Scale bars = 10 µm. Drawings by Sana Jabeen.

"*I. leptocystis*" has been applied to sequences representing at least two different species.

We compared our ITS sequences with 44 sequences retrieved from GenBank. *Inocybe dunensis* P.D. Orton (JF908262) and *I. hirculus* Vauras (AM882723) were chosen as outgroup (Fig. 5). After alignment, a 638 character long matrix was obtained, of which 352 characters were conserved, 271 variable, 194 parsimony-informative, and 76 singletons. Three major clades were formed within the ingroup sequences. *Inocybe ahmadii* clustered with *Inocybe* spp. (HG796992, JF908193) and *I. glabripes* Ricken in clade I but formed its own lineage on an isolated branch. *Inocybe leptocystis* clustered in clade II with *I. aeruginascens* Babos (labeled as "*I. bresadolae*") and *I. hystrix* (Fr.) P. Karst.

Discussion

Our analysis of sequences from *Inocybe* s.str. (clade Inocybe; Latha et al. 2016) indicated three clades I–III. Typical characters include presence of thick-walled pleurocystidia, two kinds of cheilocystidia (both pleurocystidioid cheilocystidia and clavate to sphaeropedunculate paracystidia), smooth or angular or nodulose spores with fairly large apiculi; caulocystidia and a cortina may be either present (over a variable stipe length) or absent (Kuyper 1986, as *I.* subg. *Inocybe*).

Clade I, which comprised 26 sequences split into two subgroups, is characterized by species showing a brown to pale brown or ochraceous buff colored pileus (excepting the distinctively purple brown or lilaceous *I. lanatopurpurea* Esteve-Rav. & G. Moreno), a stipe that is whitish when young then brownish, cortinate young specimens, subamygdaliform spores, and apically encrusted thick-walled pleurocystidia. Within clade I, the new species *I. ahmadii* is well separated from the other taxa, forming its own lineage with notably long branch. This phylogenetic separation is supported by its morphological differences, e.g., buff brown lamellae with pinkish tinge, a whitish stipe surface with a brownish apex, ellipsoid and phaseoliform to subamygdaliform spores, apically encrusted and narrowly to broadly lageniform pleurocystidia, and generally shorter and broader cheilocystidia. *Inocybe ahmadii* forms a subgroup with *Inocybe* sp. HG796992 (Pakistan) and JF908193 (U.S.A.) and *I. glabripes* AJ889952, AM882807 (Sweden) and JF908239 (U.S.A.), but a 86% ML bootstrap value well supports its separation from these taxa. *Inocybe glabripes* is distinguished by its olivaceous tinged ochraceous brown lamellae, its

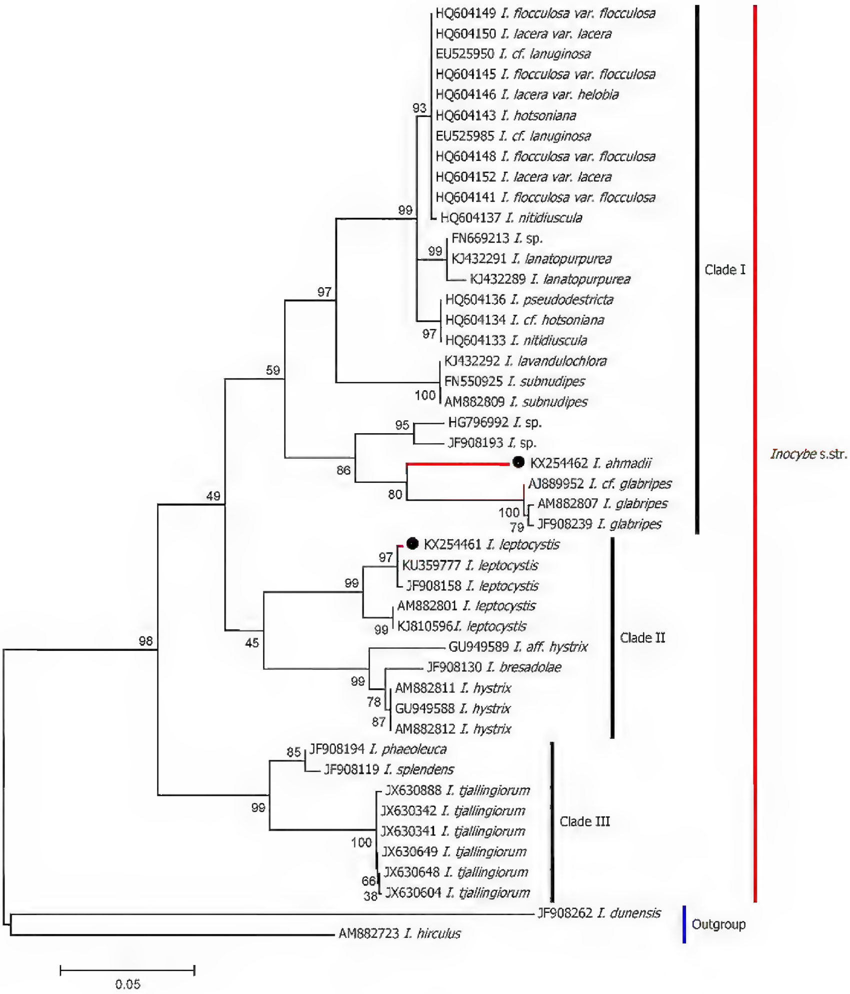
HQ604149 I. flocculosa var. flocculosa
HQ604150 I. lacera var. lacera
EU525950 I. cf. lanuginosa
HQ604145 I. flocculosa var. flocculosa
HQ604146 I. lacera var. helobia
HQ604143 I. hotsoniana
EU525985 I. cf. lanuginosa
HQ604148 I. flocculosa var. flocculosa
HQ604152 I. lacera var. lacera
HQ604141 I. flocculosa var. flocculosa
HQ604137 I. nitidiuscula
FN669213 I. sp.
KJ432291 I. lanatopurpurea
KJ432289 I. lanatopurpurea
HQ604136 I. pseudodestricta
HQ604134 I. cf. hotsoniana
HQ604133 I. nitidiuscula
KJ432292 I. lavandulochlora
FN550925 I. subnudipes
AM882809 I. subnudipes
HG796992 I. sp.
JF908193 I. sp.
● KX254462 I. ahmadii
AJ889952 I. cf. glabripes
AM882807 I. glabripes
JF908239 I. glabripes
● KX254461 I. leptocystis
KU359777 I. leptocystis
JF908158 I. leptocystis
AM882801 I. leptocystis
KJ810596I. leptocystis
GU949589 I. aff. hystrix
JF908130 I. bresadolae
AM882811 I. hystrix
GU949588 I. hystrix
AM882812 I. hystrix
JF908194 I. phaeoleuca
JF908119 I. splendens
JX630888 I. tjallingiorum
JX630342 I. tjallingiorum
JX630341 I. tjallingiorum
JX630649 I. tjallingiorum
JX630648 I. tjallingiorum
JX630604 I. tjallingiorum
JF908262 I. dunensis
AM882723 I. hirculus
93
99
99
97
97
59
100
95
86
80
100
79
49
97
99
99
98
45
99
78
87
85
99
100
66
38
Clade I
Clade II
Clade III
Inocybe s.str.
Outgroup
0.05

Fig. 5. The evolutionary history of species of *Inocybe* sensu stricto inferred by using the Maximum Likelihood method based on the General Time Reversible model in MEGA6. The tree with the highest log likelihood (–3363.8870) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are indicated above or below branches. The analysis involved 46 nucleotide sequences. There were a total of 630 positions in the final dataset. The two Pakistani collections have been labeled with ● and highlighted.

ochraceous or honey brown stipe surface, subamygdaliform spores, cylindrical to subfusiform pleurocystidia, and infrequent cheilocystidia. All these morphological characters separate *I. ahmadii* from *I. glabripes*, supporting its designation as a new species.

Clade II included 10 sequences, split into two subgroups, one of which clustered our Pakistani *I. leptocystis* sequence with four other *I. leptocystis* sequences. Species of clade II can be distinguished morphologically from clade I by their lack of caulocystidia and presence of thin-walled pleurocystidia.

Inocybe leptocystis has been reported from several different temperate regions: the U.S.A. (Atkinson 1918, Kobayashi 2002), Asia (China; KU359777, Fan & Bau, unpublished), and Europe (Austria, Kobayashi 2002; Finland, Kobayashi 2002 and Matheny 2005; Italy, Osmundson et al. 2013; Sweden, Ryberg 2009).

The sequence data suggest two different taxa are labeled as "*I. leptocystis*" within clade II: group (1) from China (KU359777) and Italy (JF908158); and group (2) from Sweden (AM882801) and China (KJ810596). Morphologically, the Pakistani collection corresponds with the descriptions of the collections from Austria and China (Kobayashi 2002), and the sequence from the Pakistani collection clusters with the group (1) sequences from China and Italy. This group (1) subclade is represented by species having an ochraceous brown to buff pileus, non-pruinose smooth stipe, thin-walled, subutriform to clavate pleurocystidia (except *I. hystrix,* which has cylindrical to sublageniform pleurocystidia).

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